

Application ser. no. 10/782,698
Attorney Docket No. W1107/20009
Amendment Dated June 8, 2008

AMENDMENTS TO THE SPECIFICATION

Please replace the title paragraph of the application, beginning on page 1, line 1 and ending on page 1, line 3, with the following title paragraph:

TITLE OF THE INVENTION:

~~[[THROMBOSPONDIN FRAGMENTS AND USES THEREOF IN]] CLINICAL ASSAYS FOR [[CANCER AND GENERATION OF ANTIBODIES AND OTHER BINDING AGENTS]]~~ THROMBOSPONDIN FRAGMENTS IN THE DETECTION, DIAGNOSIS, AND EVALUATION OF CANCER

Please replace the paragraph beginning on page 4, line 14 and ending on page 5, line 4 with the following paragraph:

In a still further related aspect, the invention is a purified and/or synthetic thrombospondin fragment or portion thereof, said fragment being one that starts between amino acid I-165 (just after the N12/I peptide) and V-263 (the start of the procollagen homology domain), inclusive, and ends between amino acid R-792 (the end of the third type 3 repeat) and Y-982 (the third of the predicted chymotrypsin cleavage sites in the C-terminal domain), inclusive. Preferably such a fragment starts between N-230 and G-253, inclusive, and ends between G-787 and V-811, inclusive, which is at or near a predicted chymotrypsin cleavage site, Y-799, in the fourth type 3 repeat; said portion being at least 3 amino acyl acids in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues). Protein molecular weights here were computed using standard computational aids (such aids are available, for example, at the web site of the Bioinformatics Organization, Inc., [[http://bioinformatics.org/sms/prot_mw.html]]; see Stothard, P. 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA

Application ser. no. 10/782,698
Attorney Docket No. W1107/20009
Amendment Dated June 8, 2008

sequences. BioTechniques 28: 1102-1104) and adjusted upwards to account for post-translational modifications. Predicted cleavage sites for chymotrypsin (and any closely related protease) were identified using tools available from the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) ~~[[See [\] and were limited to predicted sites of at least 80% probability. The uses of said fragments and portions include, but are not limited to, the induction and/or screening of an antibody and/or another binding agent of interest in a diagnostic method and use in a diagnostic assay. In particular embodiments, the invention is one of the specified fragments, rather than a portion thereof. In additional embodiments, a fragment and/or a portion can incorporate or be linked to a label and/or a carrier.](http://us.expasy.org/cgi-bin/peptidecutter/peptidecutter.pl)~~

Please replace the paragraph beginning on page 5, line 32, and ending on page 6, line 10, with the following paragraph:

4) A purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, and wherein the fragment comprises an epitope for binding at least one of the following commercially available antibodies, each of which recognizes a ~450 kDa (non-reduced) protein that is specifically identified as thrombospondin (the TSP Ab numbering, e.g., "TSP Ab-2", comes from Lab Vision Corporation, Fremont, CA ~~[[, which currently has a web site at [http://www.labvision.com/"\]\]](http://www.labvision.com/)~~ : clone designations refer to the hybridoma clone that produces a particular monoclonal antibody) It is also understood that said fragment includes a fragment that can be designed to bind a pre-existing monoclonal antibody, through the use of peptide scanning analysis, competition experiments, and other methods known in the art. It is also understood that the current invention includes, but is not limited to, uses of pre-existing antibodies independent of a purified and/or synthetic fragment, some of which uses are also listed below.

Please replace the paragraph beginning on page 11, line 16, and ending on page 11, line

Application ser. no. 10/782,698
Attorney Docket No. W1107/20009
Amendment Dated June 8, 2008

21, with the following paragraph:

All of the antibodies listed above can be purchased from Lab Vision Corporation, Fremont, CA [[currently with a web site at <http://www.labvision.com/>]]. See also the published literature such as, for TSP Ab-4, Galvin NJ et al. Interaction of human thrombospondin with types I-V collagen: direct binding and electron microscopy. *J Cell Biol.* 1987 May;104(5):1413-22). It is also understood that alternative antibodies may also be generated against any of the abovementioned epitopes.

Please replace the paragraph beginning on page 27, line 11, and ending on page 27 , line 19, with the following paragraph:

Non-antibodies also include aptamers and non-antibodies that comprise aptamers. Aptamers are DNA or RNA molecules that have been selected (e.g., from random pools) on the basis of their ability to bind to another molecule (discussed for example at the web site of the Ellington lab, in the Institute of Cellular and Molecular Biology, at the University of Texas at Austin [[, <http://aptamer.icmb.utexas.edu/>]]), wherein said molecule can be a nucleic acid, a small organic compound, or a protein, peptide, or modified peptide (such as thrombospondin or a portion thereof.). An aptamer beacon is an example of a non-antibody that comprises an aptamer (See Hamaguchi N et al.. Aptamer beacons for the direct detection of proteins. *Anal. Biochem.* 2001 Jul 15;294(2):126-131.)

Please replace the paragraph beginning on page 49, line 13, and ending on page 49 , line 27, with the following paragraph:

Gel electrophoresis is done on SDS-polyacrylamide gels (4% stacking, 10% running gel) in tris/glycine/SDS buffer (see running buffer below, pH 8.3) at 200 V/ 7-8cm at 25°C for 34 minutes. Alternative electrophoretic set-ups and procedures are well-known in the art and can be used (e.g., using gels of about 8%-12% acrylamide; omission of the stacking gel), but should reliably separate 185 kDa, 85 kDa, 50 kDa, and 30 kDa (these are the approximate apparent

Application ser. no. 10/782,698
Attorney Docket No. W1107/20009
Amendment Dated June 8, 2008

weights on a reducing gel of thrombospondin and of the three major thrombospondin fragments in plasma). Molecular weight standards were: 184 kDa, 121 kDa, 86 kDa, 67 kDa, 52 kDa, 40 kDa, 28 kDa, and 22 kDa (Fig. 3). A slightly different set of molecular weigh markers was used for Figs. 5-11, as well as human platelet thrombospondin and a recombinant thrombospondin fragment. Other molecular weight markers are suitable as well, but should include markers near to 185 kDa (the approximate weight of thrombospondin on reducing gels) and near to 85, 50, and 30 kDa (the approximate weights on reducing gel of the major thrombospondin fragments present in plasma). Suitable molecular weight standards are purchasable from a variety of commercial sources, such as Invitrogen Life Technologies [[(<http://www.invitrogen.com/>)]].

Please replace the paragraph beginning on page 50, line 4, and ending on page 50, line 22, with the following paragraph:

The fragments may be detected by the Western Blot procedure using antibodies that react with the ~115 kDa, ~55 kDa, and ~30 kDa fragments, as well as the high molecular weight fragments. As illustrative but not restrictive examples, TSP Ab-2, Ab-4, or Ab-5 monoclonal antibodies from Lab Vision Corporation (Fremont, CA [[(:<http://www.labvision.com/>)]]) can be used for this purpose (as primary antibody), as can polyclonal anti-TSP antibodies (such as Ab-8, a rabbit polyclonal antibody from Lab Vision). For more specific detection of higher molecular weight forms, TSP Ab-7 can be used. Following standard protocols, proteins from the polyacrylamide gel are transferred to a suitable membrane, unoccupied protein-binding sites of the membrane are then blocked (*e.g.*, by incubation with skim milk), and the membrane is exposed to primary antibody. The presence of TSP antibodies that have bound to thrombospondin or thrombospondin fragments on the membrane can be detected by reacting those antibodies with fluorophore-labeled antibodies against mouse IgG (secondary antibody, *i.e.*, that themselves react with the TSP Ab-4 antibodies), followed by subsequent fluorescence-based scanning of the membrane. Detection of polyclonal anti-TSP antibodies is performed similarly, using appropriate secondary antibodies. Other systems for detection of primary antibody are well-known in the art, including but not limited to other systems for labeling a

Application ser. no. 10/782,698
Attorney Docket No. W1107/20009
Amendment Dated June 8, 2008

secondary antibody, such as conjugation to an enzyme, such as horseradish peroxidase. Biotin-avidin systems are also well-known in the art, as are radioactive labeling methods.

Please replace the paragraph beginning on page 53, line 32, and ending on page 54 , line 15, with the following paragraph:

For the sandwich ELISA, one option is the use of color-coded microbeads (microspheres) with immobilized anti-TSf antibody to capture, then a fluorescent second anti-TSf antibody to detect. The detection apparatus reads each bead, one at a time, assaying for bead color as well as the signal from the second anti-TSf antibody. The advantage here is that several different analytes can be assayed at once, such as one group of beads for full-length TSP (or an epitope outside of what circulates in substantial concentration in a cancer patient) and another group of beads, of a different color, for a TSP fragment. Or, one group of beads to assay an epitope present in the larger molecular weight forms that is not present in the ~85-, ~50 kDa or ~30 kDa fragments, and another group of beads to assay an epitope present in the ~85 kDa fragment but not the ~50 kDa or ~30 kDa fragments (this is followed by a numerical calculation to yield the amounts of higher molecular weight forms, and of the ~85 kDa fragment, and of the ~50 kDa and 30 kDa fragments separately). An example of this use of color-coded beads can be found at [[<http://www.lincoresearch.com/lineoplex/technology.htm>,]]the web site for Linco Research, Inc.

Please replace the paragraph beginning on page 54, line 15, and ending on page 54, line 31, with the following paragraph:

Another option for analyzing multiple analytes is SearchLight™ Proteome Arrays, which are multiplexed sandwich ELISAs, currently adapted for the quantitative measurement of two to 16 proteins per well. It is understood herein that the method can also be used for protein fragments, such as one or more thrombospondin fragments. Using a spotting technique, 2 to 16

Application ser. no. 10/782,698
Attorney Docket No. W1107/20009
Amendment Dated June 8, 2008

target-specific antibodies are bound to each well of a microplate, although it is understood that this number can be expanded with improved spotting techniques and/or larger wells. Following a standard sandwich ELISA procedure, luminescent signals are imaged with a cooled CCD (charged coupled device) camera. The image is then analyzed using Array Vision™ software. The amount of signal generated at each spot is related to the amount of target protein in the original standard or sample. Values for specific proteins and/or protein fragments can be calculated based on the position of the spots and comparison of density values for unknowns to density values for known standards (and TSP fragments or peptides can be used as standards). The SearchLight™ technology is available through Pierce Boston Technology Center [[<http://www.searchlightonline.com/>)]], including customized arrays using proprietary reagents from outside Pierce or assay targets not currently available at Pierce [[(see http://www.searchlightonline.com/custom_array.cfm)]]]. Other technologies for multiplex assaying are also contemplated.

Please replace the paragraph beginning on page 60 , line 20 and ending on page 60 , line 29, with the following paragraph:

Figure 4 shows the results of an independent gel analysis of the samples. The samples were denatured then run on a 12% gel, transblotted, and then stained with the same TSP Ab-4 that we used before. Unlike the blot shown in Figure 3, the denaturation step here included urea, and detection used an enzymatic colorometric method that is based on horseradish peroxidase conjugates and the BioRad Opti-4CN substrate kit [[(see <http://www.discover.bio-rad.com/>)]]], not fluorescence as before. Along the left edge of lane 1, there are from top to bottom, the following handwritten numbers evident: 1, 97, 66, 45, 30, 20, and 14, respectively. With the exception of 1, the numbers correspond to the positions where standard proteins of corresponding molecular weights (in kDa) had electrophoresed.